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Regulated conformation changes in C-reactive protein orchestrate its role in atherogenesis

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C-reactive protein (CRP) is a prototypic human acute phase reactant composed of five identical subunits. Emerging evidence indicates that CRP is not merely a predictor of cardiovascular disease, but may also be a direct mediator. However, the diverse and sometimes contradictory activities of CRP have considerably hampered the attempts to define the exact role of CRP in atherogenesis. Here, we review the multiple layers of regulation of CRP's structure and function, highlighting how local inflammation conditions, such as the abundance of damaged cell membranes and redox homeostasis, can tip the balance of the pro- and anti-inflammatory activities of CRP. We propose that the highly controlled interplay between different structural conformations of CRP underlies its intrinsic property as a fine modulator of inflammation and atherogenesis.

C-reactive protein, inflammation, atherosclerosis, redox

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C-reactive protein (CRP) is an ancient protein that has been highly conserved throughout evolution [1,2]. No deficiency or coding sequence mutation of CRP has yet been identified in humans, implying that there are important biological functions for CRP. CRP is composed of five identical subunits that are non-covalently associated with each other (Figure 1). The two opposite faces of the disc-like structure of CRP present distinct binding sites for interaction with other molecules. Through the recognition face, CRP binds a panel of endogenous and exogenous ligands found on damaged cell membranes, bacterial cell walls, polysaccharides and chromatin. Ligand-bound CRP can further interact with C1q via its effector face to activate the classical complement pathway (CCP). Therefore, CRP is considered to act as a pattern recognition receptor in innate immunity, facilitating the efficient clearance of harmful materials [3]. Moreover, the effector face of CRP appears to also possess binding sites for cell surface receptors, including FcγR [4,5],

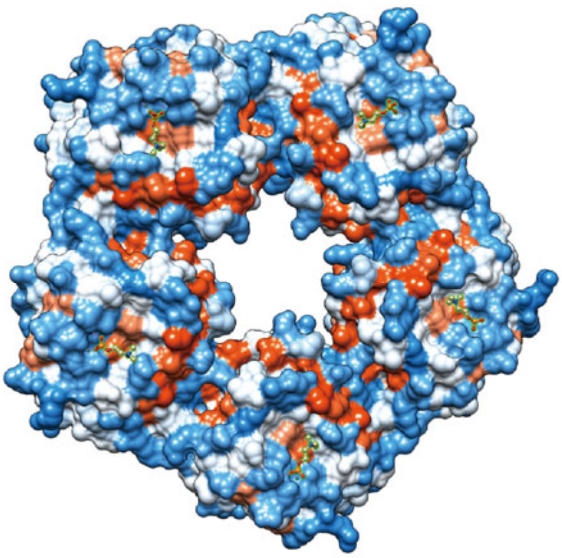

Figure 1 The X-ray crystal structure of CRP in complex with phosphocholine. Five subunits associate with each other non-covalently into a disc-like structure with a diameter of ~10 nm.

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FcαR [6], SR-A [7] and LOX-1 [8], thus giving CRP the potential to modulate the responses of immune and vascular cells. These characteristics lead to the consensus that CRP is an important component of the host defense and inflammation [1–3], despite the fact that the actual function of CRP remains to be clearly defined in an appropriate animal model [1,9,10].

CRP is a typical human acute phase reactant that is primarily secreted by the liver [1]. The serum concentration of CRP can rise more than 1000-fold in response to tissue injury or infection and returns to a baseline level of less than 1 μg/mL upon recovery of homeostasis. The tight correlation between the level of CRP and the severity of inflammation forms the basis for the broad classification of this protein as a non-specific marker of inflammation [1]. Although a concentration of CRP less than 10 μg/mL was originally considered to be clinically insignificant, accumulating evidence indicates that such a subtle elevation can be a sensitive predictor for the future risk of cardiovascular disease (CVD) in apparently healthy populations [11]. Despite considerable debates on the prediction strength [12] and causality [13] of elevated CRP levels, CRP has nonetheless gained the fame as an independent marker of CVD which may be used in the screening of high-risk individuals for primary intervention [14] or to evaluate the effectiveness of a specific treatment [15]. Furthermore, data from numerous reports describing the pro-inflammatory activities of CRP on vascular and circulating cells support the idea that CRP could also be a potential drug target for treating CVD [9,16]. These results fuel the enthusiasm for additional research but yield intense controversies as well. We will discuss these issues in the following sections.

1 The controversial role of CRP in atherosclerosis

CVD is the leading cause of death and disability worldwide. Over 17 million people died from CVD in 2008; this number will increase to 23.6 million by 2030 as estimated by WHO. Fortunately, timely intervention can effectively reduce the mortality and morbidity of CVD, which accounts for 4% and 42% of the premature deaths in high- and low-income countries, respectively. Such preventability of CVD highlights the importance of diagnosis before onset or in the early stages of the disease. Although the level of plasma cholesterol has been established as the most sensitive risk factor for CVD, nearly half of all CVD events occur in individuals with normal or low plasma cholesterol levels. Thus, it is mandatory to improve the current methods of assessing CVD risk. Because inflammation plays a critical role in all stages of atherosclerosis [17,18], the primary cause of CVD, the possibility exists that inflammation markers may also correlate with CVD progression. As the most widely used marker of inflammation in clinical practice, CRP has received intense interest and has proved to be a strong predictor of CVD independent of and complementary to plasma cholesterol levels [11,14]. Moreover, the availability of cheap, sensitive and standardized assays to measure CRP levels allows the inclusion of CRP evaluation in current methods of risk assessment and diagnosis of CVD as a reasonably cost-effective choice.

In a recent large-scale clinical investigation known as JUPITER, statin treatment reduced 5-year CVD events by 50% in apparently healthy individuals with high CRP levels but normal cholesterol levels [14]. This provides the first proof that early intervention of CVD can be effectively guided by CRP levels alone and suggests that the current methods of risk assessment will be improved by the inclusion of CRP evaluation. Interestingly, evidence has shown that changes in plaque size and the risk of recurrent events after a treatment are closely correlated with alterations in CRP levels [15,19]. Such a tight association between CRP levels and progression of the disease implies that CRP is not simply a sensitive marker but a direct mediator of CVD. Indeed, CRP can be detected in early lesions of atherosclerosis in humans [20] and its staining intensity correlates with disease development [21]. Moreover, CRP co-localizes with atherogenic mediators, including modified low density lipoproteins (LDL) [22,23], activated complement [24] and foam cells [20]. Together with the reported pro-inflammatory actions of CRP [25,26], including activation of complement and stimulation of expression of cytokines and adhesion molecules in endothelial cells, this protein is emerging as a promising drug target for CVD treatment [16]. Accordingly, a small molecule inhibitor that blocks the ligand binding site on CRP significantly reduced the complement-dependent infarction size in a rat model [27].

Despite the aforementioned compelling supportive evidence, there is also remarkable evidence that negates the role of CRP as a specific marker and a mediator of CVD. For example, several epidemiological studies failed to find an independent correlation between CRP and the disease [28–30]. A causal involvement of CRP in CVD was recently rejected by the finding that polymorphisms in non-coding nucleotides of the CRP gene affects the basal plasma levels of CRP but does not correlate with the risk of future CVD events [13]. Moreover, transgenic expression of human CRP or knock-out of endogenous CRP in atherosclerosis-prone mice has been reported to accelerate [31], show no effect [32] or even protect against the development of atherosclerosis [33,34]. Knock-in of human CRP in rabbits, on the other hand, appeared to reduce the lesion formation, albeit with no statistical significance [35]. Even worse is that *in vitro* studies frequently generate conflicting results [36–45] and some of the reported activities of CRP have been ascribed to impurities such as azide or endotoxin [39,46,47]. The acute-phase reactant nature of CRP adds an additional layer of complexity: how does a protein with two to three orders of dynamic fluctuation in its plasma levels function as a fine modulator of sophisticated cellular or physiological systems [1]?

2 The bioactivities of CRP are dependent on conformational states

These enigmas may be addressed by regulated changes in the conformation and activity of CRP that occur during local inflammation [48–53]. The pentameric assembly of CRP can be disrupted to form separated subunits that are termed monomeric CRP (mCRP) [48–53]. "mCRP" is also used as the abbreviation for "modified CRP" because the conversion to mCRP is usually accompanied by significant alterations in the native subunit structure and epitope expression. Accordingly, a significant loss of secondary structural elements and a marked relaxation of the tertiary packing have been demonstrated upon dissociation of CRP to mCRP [54–56]. Presumably due to the prominent conformation rearrangement, mCRP is unable to spontaneously re-associate back to the cyclic pentameric CRP [57]; rather, this molecule is prone to aggregating into high-order oligomers composed of tens of monomers [58]. Therefore, the propensity of self-assembly and the relative disordered/flexible conformation of mCRP, on the one hand, hinder the determination of its high-resolution structure by X-ray crystallography or NMR; while on the other hand are likely to confer mCRP enhanced bioactivities and new functionalities by presenting clusters of functional sites and by releasing critical motifs that are not accessible in the structure of CRP.

Indeed, conversion to mCRP greatly augmented the capacity of CRP to interact with various ligands and to regulate complement activation. We have shown that mCRP binds avidly to native and modified LDL, to which CRP exhibits low-to-moderate binding [59]. Moreover, we found that mCRP interacted with C1q in both the free and bound states, in contrast to CRP, which recruits C1q only when complexed with multivalent ligands [60]. As such, mCRP can either inhibit or activate the CCP depending on whether it is surface-bound. Since mCRP (but not CRP) also binds to Factor H, the activation of CCP by mCRP is largely restricted to the opsonic C3 level without proceeding to the more inflammatory C5 stage [60]. Subsequent studies confirmed the enhanced interactions of mCRP with modified lipoproteins [61] and complement [62–67] and further revealed the important contributions of mCRP in promoting non-inflammatory clearance of apoptotic or necrotic cells by controlling complement activation on the cell surface through balanced recruitment of C1q, Factor H and C4bp [64–66]. Because inappropriate handling of CRP, including lyophilization [68], storage in the absence of calcium [69] and immobilization onto microtiter wells [60,70], leads to the disruption of the pentameric structure, the reported properties of CRP suffering these technical pitfalls should be re-evaluated with Factor H interaction as a prominent example [60,64,71].

3 Dissociation of CRP localizes the enhanced bioactivities

The significantly enhanced bioactivities of mCRP suggest that mCRP is an activated isoform while CRP is in a basal state. This hypothesis thus implicates the dissociation of CRP as a key mechanism that acts as a firewall to avoid fluctuations in the serum levels of CRP to generate immediate global effects. However, the concept of mCRP has been disputed for decades since its first demonstration by Potempa et al. [54] in the early 1980s. The major concern is that the structure of the CRP molecule is exceptionally stable in the presence of calcium [1] and the preparation of mCRP requires highly denaturing treatments of CRP with concomitant chelation of calcium [54]. Therefore, the identification of dissociation pathways with a pathophysiological relevance is of paramount importance. The first hint came from the open-ring-like appearance of CRP bound to lipid monolayers when visualized by electron microscopy, arguing for a membrane-induced dissociation [72]. With this observation, we have provided compelling evidence that the lysoPC-dependent, multi-point binding of CRP to model or apoptotic cell membranes can overcome the stabilization effects of calcium and induce a rapid formation of a hybrid state [73]. This hybrid state, in the form of a separated subunit with near-native conformation, is termed $mCRP_m$ and eventually gives rise to mCRP with the aid of the relatively hydrophobic and fluidic characteristics of membranes [73].

The five subunits in CRP, in principle, provide multiple binding sites in one molecule. This is of particular importance for the effectors and receptors of CRP whose activation usually requires clustering or multi-point attachment. Among those, C1q, a hexameric protein, is an outstanding representative. At least two of the C1q subunits need to be in a bound state in order for C1q to be activated, resulting in initiation of the CCP. Since the relatively small size of CRP (~10 nm in diameter) allows it to bind only one C1q subunit, this leads to the idea that close packing on the surface is the prerequisite for CRP to activate the complement pathway [74]. As such, the low expression level of CRP under normal physiological conditions and during the early phases of inflammation would restrict its major contribution to the late stages of tissue injury or infection. This appears to be incompatible with the proposed function of CRP in the non-inflammatory clearance of apoptotic cells in times of health and disease [75] and its role as a component in the innate immunity and host defense [1,3]. In this regard, the rapid formation of $mCRP_m$ can override the size limitation such that even the dissociation of a single CRP molecule can provide multi-point binding to, for example, activate

C1q. Therefore, the damaged membrane-induced stepwise dissociation of CRP can represent an efficient mechanism for prompt amplification of the bioactivities locally on membranes, as evidenced by the significantly enhanced CCP activation by $mCRP_m$ [73].

Due to the reduced binding affinity associated with the loss of multi-point attachment as the result of monomerization, $mCRP_m$ can detach from the membrane and undergo further conformational changes leading to the generation of extracellular mCRP [73], which may later reassociate with the membrane by direct insertion. Alternatively, analogous to the dissociation of CRP induced by immobilization onto plastic surfaces, the mild hydrophobicity of damaged membranes can also facilitate structural rearrangements in $mCRP_m$ for direct conversion to membrane-associated mCRP. However, considering the relatively slow dynamics of the conversion process, the significant accumulation and effective actions of mCRP mostly likely occur in inflammatory loci where injured cells and damaged membranes are enriched. This has led us to propose that the dissociation of the CRP molecule is an activating mechanism that is required for the expression of enhanced bioactivities and is also a buffering mechanism that localizes the actions of mCRP into inflammatory loci to prevent the possible global effects directly induced by large-scale alterations in the serum levels of CRP [73]. Consistent with our proposal, immunohistochemical analyses using highly specific antibodies revealed that mCRP, not CRP, was the major isoform present in local lesions, including atherosclerotic plaques [55,76], diabetic kidneys [77] and stroke neovessels [78].

The proposal of disturbed membranes being the major sites for mCRP generation has been further corroborated by elegant studies that demonstrate the binding and dissociation of CRP on the surface of necrotic cells [65,66] and activated platelets [76,79] in a lysoPC-dependent manner [76]. Moreover, Dr. Peter and colleagues [80] recently identified novel pathways for mCRP generation upon binding of CRP to amyloid aggregates, the typical pathological feature found in brains of persons with Alzheimer's disease, and lysoPC-enriched microparticles released by activated cells or isolated from blood of patients with myocardial infarction [81]. As disturbed membranes, these newly identified inducers also present multi-point binding sites and relative hydrophobicity, factors critical for driving the conversion of CRP to mCRP [73]. An additional factor, high membrane curvature, has recently been revealed by showing that CRP preferentially binds and dissociates on lipid-coated nanoparticles smaller than 28 nm [82]. This is likely due to the less dense packing of lipid head-groups in the context of nanoparticles that lack stuffing lipids (e.g., cholesterol) and proteins found in biological membranes, which would increase the accessibility of PC ligands for easier binding of CRP, and due to the stronger tension imposed to facilitate dissociation when more than one subunit in a single pentamer is bound by the particle. However, further reduction of the particle size to below 20 nm appeared to be unfavorable for CRP dissociation [59], which may relate to the difficulty in multi-point binding of CRP to smaller particles.

All of the dissociation pathways of CRP identified thus far are primarily associated with local inflammatory conditions, such as damaged membranes [65,66,73,76,79], pathological protein aggregates [80] and microparticles released by activated cells [81]. Interestingly, the acidic pH frequently found in inflamed tissues can also boost the binding capacity of CRP even in the absence of pentamer dissociation [83]; however, after binding to immobilized ligands at a low pH, a prominent exposure of the mCRP epitope has been observed [83]. These results further support the idea that interactions of CRP with the inflammatory microenvironment are critical for the expression of enhanced bioactivities. In addition to the localized dissociation of CRP, direct synthesis by activated macrophages/monocytes likely provides another source of mCRP [84]. As such, CRP may primarily serve as a systemic inflammation marker, while mCRP is the active player in local inflammation. Nonetheless, if locally formed mCRP could be released into circulation, it might serve as a better marker for the underlying inflammatory process than CRP. Accordingly, mCRP has been detected on circulating microparticles [81] and the discovery of RNA aptamers specifically recognizing mCRP [85] may eventually result in the development of clinical assays for mCRP. Alternatively, autoantibodies against mCRP that were found to be correlated with autoimmune [86–88] and heart diseases [89] could be regarded as an indirect index to measure global mCRP production.

4 Redox regulation of bioactivities of monomeric CRP

The actions of mCRP in various aspects of inflammation are emerging; however, the mechanism by which the potent activities of mCRP are regulated remains poorly defined. By careful examination of the sequence of human CRP, we found that there are two cysteines (i.e., Cys36 and Cys97) that form an intra-subunit disulfide bond. Their evolutionary conservation strongly suggests that these cysteines are essential for the structure and function of CRP. We further showed that this intra-subunit disulfide bond could be reduced only in mCRP but not in CRP. This pattern of reduction indicates that the dissociation of CRP is the prerequisite for the disulfide bond to be modulated by the environmental redox status. In addition to physiological small reducing agents, thioredoxin, a ubiquitous reducing enzyme that is critical for thiol/disulfide redox homeostasis [90,91], was also found to reduce mCRP levels with high efficiency. Indeed, the frequent co-localization of thioredoxin and mCRP in advanced plaques indicates that the reduction of mCRP could occur in local inflammation. After reduction, mCRP showed moderately enhanced capacity to activate the CCP and significantly enhanced capacity to induce pro-inflammatory responses on endothelial cells (EC) in rabbit aorta and in mice. Comparable results were obtained with Cys-mutated mCRP, in which the two cysteines were mutated to alanines. These findings thus indicate the intra-subunit disulfide bond as an important switch modulating the activities of mCRP [55].

Although FcγRIII has been reported to be the putative receptor for mCRP on neutrophils [56], we failed to find any impact of this receptor on EC binding and activation of Cys-mutated mCRP. By using a combination of experimental techniques, we have demonstrated that Cys-mutated mCRP interacts with model and EC membranes by direct insertion into the cholesterol-enriched lipid raft microdomains [92]. Moreover, disruption of lipid rafts by MβCD or nystatin eliminated the stimulation effects of Cys-mutated mCRP on EC and in rabbit vessels. Accordingly, we identified a cholesterol binding motif (CBM; a.a. 35–47) containing Cys36 in the sequence of human CRP which has been conserved throughout evolution. Indeed, reduction or mutating cysteines promoted the interactions of Cys-mutated or reduced mCRP with both model and cell membranes. In addition, the specific antibody 8C10 against the epitope containing the CBM markedly repressed lipid raft insertion of Cys-mutated mCRP. These lines of evidence thus identify lipid rafts as the major cellular sensors of reduced mCRP [92] and reveal that the unlocking of CBM in mCRP after reduction underlies the enhanced capacity of reduced mCRP to interact with lipid rafts and to stimulate cellular responses [55]. Interestingly, rabbit mCRP also showed enhanced capacity to activate EC after reduction [55]. This observation provides evidence that cholesterol binding and redox regulation of mCRP are functionally conserved.

Lipid rafts are specialized signaling platforms in the plasma membrane [93]. Hence, interacting with lipid rafts confers mCRP unique advantages to access to various signaling components. For example, integration into a lipid raft may enable mCRP to interact with transmembrane segments of membrane proteins that are inaccessible from the extracellular space. Moreover, membrane insertion-induced conformation changes in mCRP, which may not be the same for reduced and non-reduced counterparts, likely create additional binding specificities for surrounding receptors. As such, mCRP could further act as a scaffolding protein due to its polymeric assembly, triggering distinct downstream pathways under different conditions by organizing diverse sets of co-factors. Accordingly, disruption of lipid rafts has recently been shown to reverse the effects of mCRP in promoting monocyte adhesion to fibrinogen [76] and in augmenting fibrin clot formation on EC [94]. Since the lipid raft insertion and cell stimulating capacities of mCRP are affected by the redox state, care should be taken to control the experimental conditions, including the use of wild-type or Cys-mutated mCRP, the prevalence of lipid rafts in certain cell types, and the contents of reducing agents in cell culture media. In addition, our unpublished results indicate that freshly prepared wild-type mCRP, membrane-associated mCRP/mCRP$_m$ and spontaneously dissociated mCRP in solution-phase are more prone to reduction.

5 Concluding remarks

Taken together, we can form a revised cascading model demonstrating how the controlled interplay between different CRP isoforms can contribute to the development of CVD (Figure 2). There are at least three distinct structural and functional states of CRP: CRP, mCRP and reduced

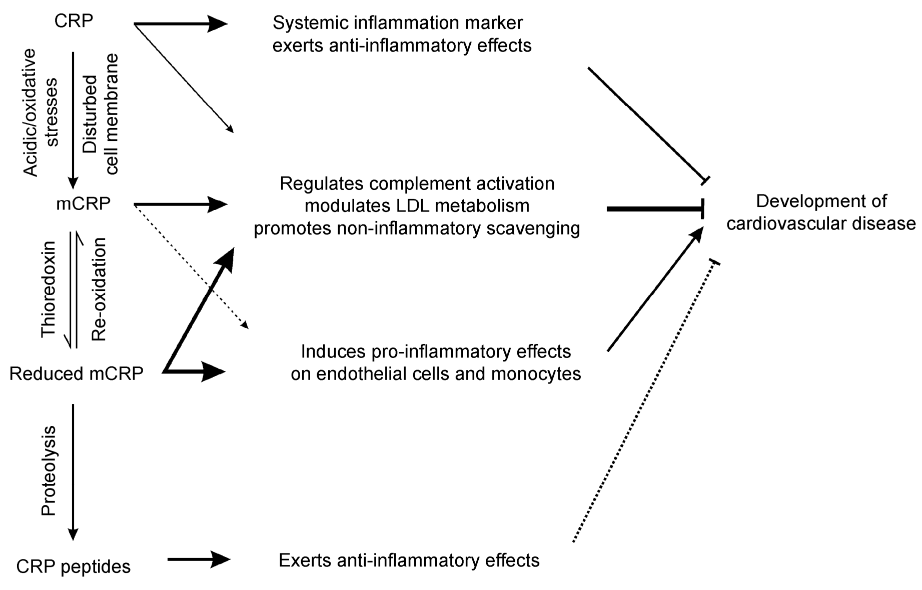

Figure 2 A cascading model for the function and regulation of CRP isoforms in cardiovascular disease.

mCRP. CRP produced by the liver appears to primarily serve as a systemic inflammation marker with some anti-inflammatory activities [53]. In inflammatory loci, CRP molecules either synthesized by local extra-hepatic cells [95–99] or entered into these loci from circulation can dissociate into mCRP with the aid of damaged cell membranes and acidic stresses. mCRP may mainly act as a pattern recognition molecule to facilitate the mobilization of the innate immunity and the clearance of damaged materials. mCRP can be further reduced to express the enhanced cell stimulating capacity. Although the reduction of mCRP in the oxidative inflammatory loci seems to be paradoxical, it is worth noting that extracellular thioredoxin is up-regulated in atherosclerotic plaques [100] and is an emerging marker of inflammation [90,101]. Moreover, the generation of mCRP on membranes favors local accumulation of mCRP and its subsequent reduction by secreted and/or membrane-associated thioredoxin [102] or other reductases, thereby allowing reduced mCRP to be functional *in situ*. The potent activities of reduced mCRP could be negatively regulated tiple by auto-oxidation of the disulfide bond and proteolysis. Interestingly, the degraded CRP fragments are also biologically active [103].

This model highlights the complexity of CRP regulation and raises the concern that the interpretation of the role of CRP in CVD should take into account the highly context-dependent interplay between the different structural states of CRP isoforms. Indeed, dissociation of CRP into mCRP significantly enhances the ligand interaction capacity. Irrespective to its redox state, mCRP can regulate LDL metabolism [59,104] and opsonic activation of the complement pathway [60], likely exerting atheroprotective actions, in particular during the initial stages of atherogenesis [105] by promoting the clearance of the damaged material. Further reduction of the intra-subunit disulfide bond in mCRP unlocks the CBM, thereby unmasking pro-inflammatory activities, which may accelerate disease progression and trigger acute events. This result is also the first demonstration of a sulfur switch (i.e., Cys36), an essential component of redox regulation [106], in an acute phase reactant, expressing an “on” state in the reduced form. Such a cascading mechanism would ensure prompt and localized responses and determine the expression of anti- or pro-inflammatory activities of CRP, mCRP and reduced mCRP during inflammation. As such, these multiple layers of regulation underlie the intrinsic competence of CRP as a fine modulator of inflammation through sequential conformational changes.

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